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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Multiple system atrophy (MSA) is a progressive neurological disorder characterized by parkinsonism, cerebellar dysfunction and autonomic impairment. The cardinal pathological feature of MSA is the glial cytoplasmic inclusion (GCI) in oligodendrocytes, and a major component of GCIs is α-synuclein. We identified a rat oligodendrocytic precursor cell line (CG-4) that produces α-synuclein and characterized α-synuclein in the cells and production of α-synuclein after differentiation to mature oligodendrocytes and astrocytes. Studies to date suggest that oxidative stress can cause aggregation of α-synuclein. We have further developed CG-4 cells that can overexpress human α-synuclein constitutively or under the control of a tetracycline inducible promoter.

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INTRODUCTION

Multiple system atrophy (MSA) is a progressive neurological disorder characterized by parkinsonism, cerebellar dysfunction and autonomic impairment. The cardinal pathological feature of MSA is the glial cytoplasmic inclusion (GCI) in oligodendrocytes, and a major component of GCIs is α-synuclein. We identified an oligodendrocytic precursor cell line, CG-4, that constitutively produces α-synuclein. We characterized α-synuclein-immunoreactive (α-synuclein-IR) material in the CG-4 cells (with attention to higher molecular weight forms), studied the effects of oxidative stress, produced CG-4 cells that constitutively overexpress human α-synuclein and more recently have produced CG-4 cells that express human α-synuclein under control of the tetracycline-ON (inducible promoter) system.

BODY

1. Characterization of CG-4 Cells

Maintenance and differentiation of the cells - CG-4 cells were kindly provided by Dr. R. H. Quarles (NIH). The cells were maintained in cell growth medium consisting of 70% Dulbecco's modified Eagle's media with N-1 supplement (DMEM-N1media) plus 10 ng/ml biotin and 30% B104 conditioned media (Louis et al., 1992). To differentiate the CG-4 cells into oligodendrocytes, the cells were washed twice with Hank's balanced salt solution (HBSS) and maintained in DMEM-N1 media plus 10ng/ml biotin for 6 days. After 48 hr, 0.1% fetal calf serum was added to the media to promote survival of differentiated oligodendrocytes. To differentiate CG-4 cells into astrocytes, CG-4 cells were washed twice with HBSS and 20% fetal calf serum was added to the DMEM-N1 media. Stable human α-synuclein transfected B103 cells and vector transfected B103 cells were used as control cells (Takenouchi et al., 2001).

Immunohistochemistry - Cells were seeded onto poly-L-ornithine coated coverslips and maintained under undifferentiated condition or differentiated condition, as previously described. The cells were fixed with 4% paraformaldehyde for 20 min. After three rinses in PBS, the cells were incubated in PBS/0.25% Triton X-100/20% normal goat serum for 1 hr. For single-labeling, the cells were incubated in monoclonal anti-synuclein-1 (1:100, Transduction Lab.) overnight at 4°C. After washing with PBS/0.25% Triton X-100 three times, the cells were incubated in biotin-conjugated anti-mouse IgG (Jackson Immunoresearch Lab.) for 1 hr and rinsed. The cells were then incubated with Cy3-streptavidin (Jackson Immunoresearch Lab) for 1 hr. The coverslips were washed three times in PBS and mounted.

For double-labeling, the cells were first incubated in cell specific antisera, either anti-A2B5 (1:200, Chemicon), anti-myelin basic protein (MBP) (1:800, a gift of Dr. John Whitaker), or anti-glial fibrillary acidic protein (GFAP) (1:200, Chemicon) for 1 hr. After washing, the cells were incubated in the corresponding secondary antibodies, FITC-conjugated anti-mouse IgM (JacksonImmuno) for anti-A2B5, FITC-conjugated anti-

rabbit IgG (1:100, JacksonImmuno) for anti-MBP or anti-GFAP, for 1 hr. Anti-syn1 staining described above was followed after washing with PBS/0.25% Triton X-100 three times.

Preparation of cell lysate - Cells were washed with PBS twice and lysed with ice-cold lysis buffer (1 mM Hepes, 5 mM benzamidine, 2 mM 2-mercaptoethanol, 3 mM EDTA, 0.5mM magnesium sulfate, 0.05% sodium azide and 10 μg/ml leupeptin). The cells were sonicated using a ultrasonic processor three times for 5 sec on ice. The cell lysate was separated by centrifugation for at 100,000 x g at 4°C for 1 hr into supernatants (cytosolic fraction) and pellets (particulate fraction). The pellets were resuspended with the lysis buffer described above. Protein level in both cytosolic and particulate fraction was determined by BCA protein assay (Sigma).

Immunoblotting - Proteins were separated by SDS-PAGE, and transferred onto nitrocellulose membranes as previously described (Hashimoto et al., 1999). The blots were blocked with 3% BSA in Tris buffered saline (TBS) containing 0.2% Tween (T-TBS) and NP-40 for 30 min. After blocking, the blots were incubated overnight at 4°C with monoclonal anti-α-synuclein antibody (1:1000, Transduction Lab). The blots were washes with T-TBS containing 0.1% NP-40 three times and incubated with peroxidase conjugated anti-mouse IgG (1:10,000, JacksonImmuno.) for 1.5 hr. After washing three times with T-TBS containing 0.1% NP-40, immunoreactive bands were detected with enhanced chemiluminescence (Amersham).

Two-dimensional gel electrophoresis - Duplicate two-dimensional electrophoresis was performed by Kendrick Labs Inc (Madison, WI) as follows: Isoelectoric focusing was carried out in glass tubes of inner diameter 2.0 mm using 2% pH 3.5-10 (Amersham PharmaciaBiotech). One μg of an isoelectric focusing internal standard, tropomyosin, was added to the sample. After equilibration for 10 min in Buffer'O' (10% glycerol, 50 mM dithiothreitol, 2.3% SDD and 62.5 mM Tris, pH 6.8), the tube gels were sealed to the top of the stacking gel of 10% acrylamide Tris-HCl gel and SDS-PAGE was carried out.

Mass Spectrometry analysis - One of the duplicate gels was stained with Coomassie Blue and the other one was transferred to PVDF membrane. The blot was stained with Coomassie and scanned on a desk-top scanner. Then the blot was immunoblotted with anti-α-synuclein (Transduction Lab) as described before. Coomassie blue-stained spot was cut out from 2D gel corresponding the immunoblot and digested with trypsin. The resulting peptide mixture was subjected to MALDI-MS at the Protein Chemistry Core Facility at Howard Hughes Medical Institute (Columbia University, New York, NY).

Alpha-synuclein endogenous expression in CG-4 - In order to investigate the expression of α-synuclein in CG-4 cells, cytosolic fraction and particulate fractions of CG-4 cells were compared with those of human α-synuclein transfected B103 and vector transfected B103 described previously (Takenouchi et al., 2001). In CG-4 cells, endogenous α-synuclein-IR material, which migrated with our positive controls (rat brain homogenate and recombinant human α-synuclein and rat brain homogenate), was

detected in the cytosolic fraction cells, while it was barely detected in vector transfected B103. However, the amount of α-synuclein-IR material was rather moderate when compared with that of human α-synuclein transfected B103. In all three cell lines, intense 46kD α-synuclein-IR material was observed in both cytosolic and particulate fractions. 33kD α-synuclein-IR material in the particulate fraction distinguished CG-4 cells from vector transfected B103. Further analysis of α-synuclein expression and distribution of CG-4 cells were performed by immunolabelling. Alpha-synuclein-IR materials were diffusely distributed in cell bodies of CG4 cells. Alpha-synuclein immunoreactivity was more abundantly observed in human α-synuclein transfected B103 but not in vector transfected B103. No synuclein-IR inclusions were observed in any cells. Control experiments were carried out by omitting the primary antibodies, and faint, nonspecific labeling was observed.

Characterization of high MW of α-synuclein-IR material in CG4 -To characterize the 46kD α-synuclein-IR material, duplicate 2D SDS-PAGE was performed, followed by Coomassie blue staining or immunoblotting. The 46kD α-synuclein-IR material had basic pI, while 20kD α-synuclein-IR material had acidic pI. Using MALDI-MS, the proteins in the Coomassie-stained spot corresponding to 46kD α-synuclein-IR material were identified as the mixture of β-tubulin, γ-enolase, and AK003217. There was no distinct Commasie-stained spot corresponding 20kD α-synuclein-IR material. We further investigated the possibility that the identified proteins cross-react with anti-α-synuclein antibody. Although γ-enolase and β-tubulin migrated at 46kD in 1D SDS-PAGE, the intense 46kD α-synuclein-IR band observed in cytosolic fraction of CG-4 cell (total 25 μg protein) was not observed in γ-enolase (2.5 μg) nor β-tubulin (10 μg). Since AK003217 is not commercially available, the possibility if cross-reactivity with anti α-synuclein antibody could not be completely examined.

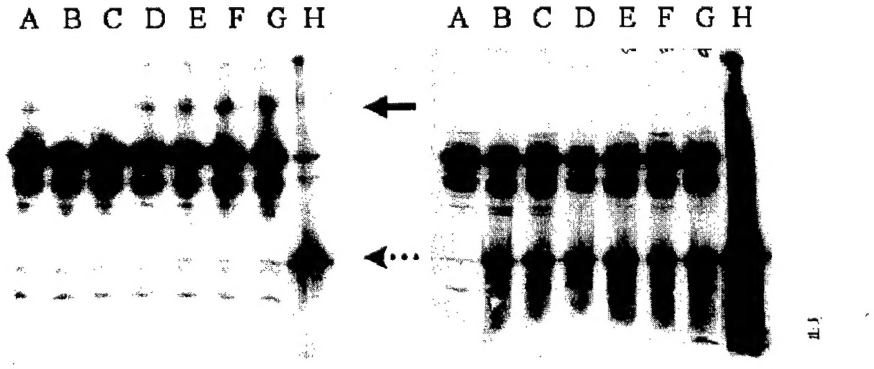
Alpha-synuclein expression after differentiation to oligodendrocytes - To investigate the expression of α-synuclein in CG-4 cells after differentiation, CG-4 cells were differentiated into an oligodendrocytic phenotype or an astrocytic phenotype and compared with CG-4 cells in undifferentiated condition. Most of CG-4 cells in undifferentiated condition were labeled with the O-2A progenitor marker, anti-A2B5. Alpha-synuclein was diffusely distributed in the cytoplasm of A2B5-positive cell. After differentiation into an oligodendrocytic phenotype, we confirmed that CG-4 cells were labeled with mature oligodendrocyte marker, anti-MBP. Alpha-synuclein was distinctively expressed in the cell bodies and processes of MBP-IR cell. After differentiation into astrocytic phenotype, we confirmed that CG-4 cells were labeled with the astrocytic marker, GFAP. In GFAP-IR CG-4 cells, α-synuclein was diffusely distributed in the cell bodies.

Alpha-synuclein expression in CG-4 cells after differentiation was also compared by immunoblotting. In cytosolic fraction, α-synuclein-IR material was expressed more in oligodendrocytic differentiated CG-4 cells than in undifferentiated CG-4 cells and astrocyte differentiated CG-4 cells. The intense 46kD α-synuclein-IR band was remarkably faint in oligodendrocyte differentiated CG-4 and astrocyte differentiated CG-

4, when compared with undifferentiated CG-4. In particulate fraction, the change of 46kD α-synuclein-IR band was not distinct. In astrocyte differentiated CG-4 cells, 33kD α-synuclein-IR band was observed in both cytosolic and particulate fractions.

2. Effects of oxidative stress

We have studied the effects of a number of oxidative treatments on aggregation of α-synuclein. To date treatment we have not found that treatment with $FeCl_2$ and H_2O_2 individually caused aggregation. In the study illustrated below we noted that treatment of the CG-4 cells with both $FeCl_2$ and H_2O_2 for 48 hours increased the aggregation of α-synuclein. Immunoblots of the particulate fraction (left) and cytosolic fraction (right) fractions from cell lysates cells were labeled with antibody (Transduction) raised against rat α-synuclein: lane A - B104 neuroblastoma cells; lanes B-G - CG-4 cells; lane H - rat brain homogenate. The CG-4 cells had been treated for 48 hours with 200 μM $FeCl_2$ (lanes C-G) and H_2O_2 (lanes D-100 μM, E-200 μM, F-300 μM, G-400 μM). The dashed arrow indicates α-synuclein monomer. The solid arrow indicates a α-synuclein-IR material at a higher molecular weight, which increases with increasing concentration of H_2O_2 and is consistent with aggregation of α-synuclein.



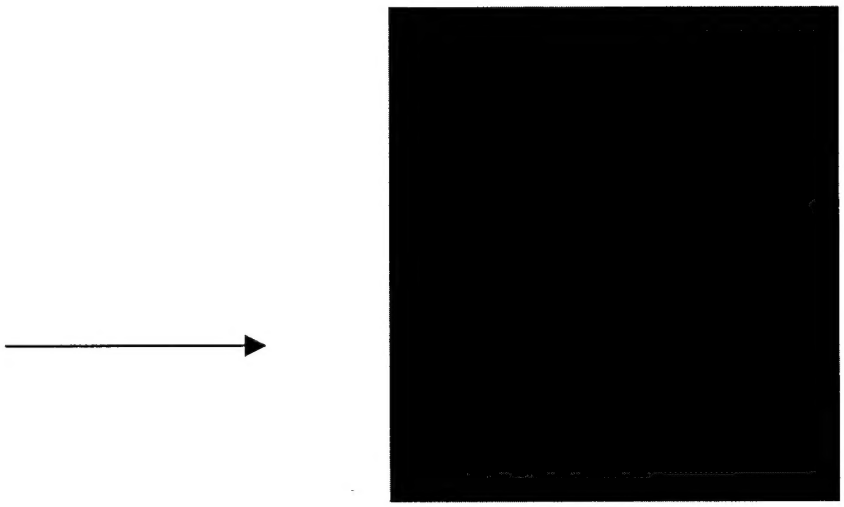
In normal CG-4 cells, we have not found that treatment with the nitration agent S-nitrosylglutathione or with the proteasome inhibitor lactacystin resulted in obvious aggregation.

3. Constitutive overexpression of α-synuclein human α-synuclein overexpressing CG-4 cell

pLNCX2 retroviral vector and α-synuclein cDNA were digested with appropriate restriction enzymes and purified after treating with phosphatase. The digested vector and α-synuclein gene fragment were ligated and transformed into E. coli. Alpha-synuclein-pLNCX2 retroviral vector was identified by restriction analysis and confirmed orientation and junctions by sequencing. Plasmid manipulation was performed at the lab of my collaborator, Dr Masliah.

Echo Pack 2™-293 packaging cells were seeded in 60 mm tissue culture and allowed to grow 70% confluence. Ten μg of plasmid DNA (pLNCX2-α-synuclein-retroviral vector) was used to transfect 60-mm plate, using Superfect (Qiagen). Culture medium was aspirated 2.5 hr after transfection and 3 ml of complete medium was added. To increase viral titer, cultures were incubated for 48 hr.

CG-4 cells were plated at a cell density of 1-2 x 10^5 per 60-mm plate 12-18 hr before infection. Medium from packaging cells was collected and filtered through a 0.45-μm cellulose acetate or polysulfonic filters. Virus was added to target cells, and polybrene was added to culture medium. Medium was replaced with fresh medium after 24 hr of incubation. The infected cells were used for experiments or for selection as soon as possible, but no earlier than 24 hr after the last infection. The infected cells were selected with G418 48 hours after infection. Large, healthy colonies were isolated using cloning cylinders and maintained. Twenty one colonies were screened by immunoblotting and immunolabelling.



A B C D E

Immunoblots have been immunolabelled with the antibody LB509, which recognizes human α-synuclein but not rat. A. CG-4 cells. B, C, D separate cell lines that have been genetically engineered to overexpress human α-synuclein. E recombinant human α-synuclein. Arrow indicates human α-synuclein.

4. Expression of human α-synuclein under control of the tetracycline-inducible promoter

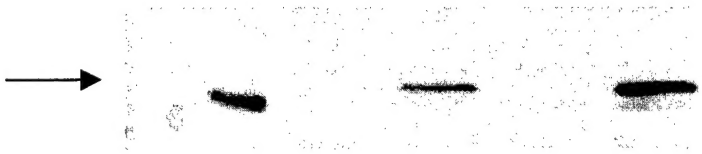
Virus production - Echo pack 2 packaging cells were transfected with pRevTet-On vector, pRevTRE- α-synuclein vector or pRevTRE-Luc vector, as described above. Virus containing media were collected 48 hr later and filtered with 0.45 μm filter.

Stable Tet-On CG-4 cells - CG-4 cells were infected with virus-containing medium (RevTet-On) as described above. Two days after infection, the cells were

treated with G418. Once colonies were visible, healthy colonies were isolated and transferred to individual wells. To identify G418 resistant clones that have high inducibility and low background, each clone was infected with RevTRE-Luc. Luciferase assay was performed to identify appropriate clones. Clones with highest fold-induction were selected and expanded.

Double-stable, inducible CG-4 cells Stable Tet-On CG4 cells were infected with RevTRE-α-synuclein virus, as described above. After selecting clones with hygromycin (the cells also carry the gene for hygromycin resistance), 30 clones were isolated.

We are now screening the clones for production of human α-synuclein after exposure to doxycycline and so far two have been screened and both showed CG-4 cell lines in which human α-synuclein is expressed under the control of the Tet-ON system.



A B C D E

A. Molecular weight marker. B -TET-ON- RevTRE-α-synuclein CG4-1 no treatment; C - TET-ON- RevTRE-α-synuclein CG4-1 treated for 48 hours with doxycycline (2 μg/ml); D - TET-ON- RevTRE-α-synuclein CG4-2 no treatment; E - TET-ON- RevTRE-α-synuclein CG4—2 treated for 48 hours with doxycycline (2 μg/ml). Arrow indicates human α-synuclein.

KEY RESEARCH ACCOMPLISHMENTS

1. Characterization of α-synuclein-IR material in an oligodendrocytic cell line (CG-4). This thorough characterization will allow investigators in MSA research to utilize the CG-4 cell line for research in MSA.

2 Production of CG-4 cells that overexpress human α-synuclein constitutively or express it in a controlled fashion under the control of the tet-ON system.

3. To date our research suggests that oxidative stress can cause aggregation of α-synuclein in oligodendrocytic cells. We are in the process of extending these results in cells that overexpress human α-synuclein.

REPORTABLE OUTCOMES

The above work will be presented at the 2002 Annual Meeting of the Society for Neuroscience in two presentations.

Tsuboi K, Hashimoto M, Masliah E, Shults CW. Alpha-synuclein immunoreactive materials in oligodendrocytic progenitor cells.

Shults CW, Hashimoto M, Masliah E, Tsuboi K. Stable human alpha-synuclein overexpressing oligodendrocytic progenitor cells.

Manuscripts will be prepared from the work described in these abstracts.

CONCLUSIONS

Alpha-synuclein occurs in an oligodendrocytic precursor cell line and persists when the cells are differentiated to oligodendrocytes and astrocytes. Under certain conditions, oxidative stress can favor aggregation of α-synuclein in oligodendrocytic cells. It is possible to develop oligodendrocytic cells that overexpress α-synuclein both constitutively and under the control of the tet-ON system.

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